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Immunohistochemical activity of Prohibitin-2 and Stomatin-Like Protein-2 in patients with ulcerative colitis

BOWEL

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Original Article

ABSTRACT

Background/Aims: We aimed to semi-quantitatively investigate prohibitin-2 (Phb-2) and stomatin-like protein-2 (Slp-2) expressions in patients with ulcerative colitis (UC) and healthy controls using the immunohistochemical (IHC) method. We also aimed to evaluate the correlations between the activity of UC and the expressions of these two proteins.

Materials and Methods: Ninety-five patients with UC (82 males and 13 females) and 38 healthy controls (35 males and 3 females) were included. Clinical and endoscopic activities of UC were assessed. Conventional laboratory activation parameters and severity of inflammation measures were used for the evaluation of histological activity. IHC staining of biopsy samples for the two proteins were semi-quantitatively applied, similar to previously described methods for colon adenocarcinomas.

Results: IHC scores of Phb-2 were lower but Slp-2 scores were higher in the UC group than in the healthy controls ($p<0.05$ and $p=0.003$, respectively). Phb-2 scores were positively correlated with clinical and histological activities ($r=0.364$, $p<0.05$ and $r=0.220$, $p<0.032$, respectively). In the UC group, endoscopic activity scores, C-reactive protein levels, and sedimentation rates were also positively correlated with Phb-2 scores ($r=0.279$, $p<0.05$, $r=0.216$, $p<0.05$, and $r=0.216$, $p<0.05$, respectively). IHC scores of Slp-2 were not significantly correlated with the activity parameters of UC. However, there was a significant positive correlation between the expressions of Phb-2 and Slp-2 proteins ($p<0.001$).

Conclusion: Phb-2 may serve as a valuable new biomarker for predicting the severity of all UC activity parameters. The therapeutic effectiveness of both Phb-2 and Slp-2 should be taken into consideration.

Keywords: Immunohistochemical activity, prohibitin-2, stomatin-like protein-2, ulcerative colitis

INTRODUCTION

Until recently, the pathogenesis of ulcerative colitis (UC) has not been entirely understood. Uncontrollable inflammation and oxidation on colonic epithelial cells cause clinical and pathological consequences (1). Currently, pathogenesis-based studies in UC are gaining interest.

Prohibitin (Phb)-2 and stomatin-like protein-2 (Slp-2) are structural proteins that belong to the Stomatin/Prohibitin/Flotillin/HflC/K/C protein family. Both proteins are mainly located in the mitochondrial inner membrane of cellular membranes. Two types of prohibitins have been identified: Phb-1 and Phb-2. These two are homologous and form a complex in the mitochondrial inner membrane (2). Phb is an evolutionarily conserved protein and exhibits a remarkable degree of sequence similarity across species: rat Phb is different from the human protein sequence by only a single amino acid (3). Slp-2 coalesces with cardiolipin, and regulates the optimal assembly of prohibitins and other oxidative enzymes in the mitochondrial inner membrane. Slp-2 is located in the T-cell membrane receptor and modulates immunologic activity (4-7). Mitochondrial dysfunction is an important pathogenic factor that aggravates oxidative damage in UC. Deficiency of Phb-2 and Slp-2 may lead to mitochondrial dysfunction (2,4,5).

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Phb exerts anti-inflammatory and anti-oxidative activities in intestinal epithelial cells. In human studies, it has been shown that Phb expression is decreased in the colonic mucosa of patients with inflammatory bowel disease compared to that of healthy controls. It has also been suggested as a therapeutic agent in experimental colitis models (2,8-11). Increased levels of Phb-2 and Slp-2 are related to poor prognosis in different types of malignancies (12-15). The role of Slp-2 has not been entirely elucidated thus far in inflammatory bowel diseases.

In this research, we aimed to semi-quantitatively investigate the expressions of Phb-2 and Slp-2 proteins in patients with UC and healthy controls using the immunohistochemical (IHC) method. Relations between clinical, histologic, endoscopic, and laboratory activities of UC and the IHC indices of these proteins, as well as the relationship between Phb-2 and Slp-2 proteins, were evaluated.

MATERIALS AND METHODS

Between November 2012 and June 2013, 95 patients with UC (82 males and 13 females) and 38 healthy controls (35 males and 3 females) who were admitted to our gastroenterology outpatient clinic were enrolled in the study (Table 1). The study was approved by the Local Ethics Committee of GATA Haydarpaşa Training Hospital and written informed consent was obtained from all participants. Volunteers with severe organ failure, malignancies, acute or chronic inflammatory diseases, gut resection, and other participants who had contraindications for colonoscopy were excluded from the study. In the UC group, both newly or previously diagnosed patients were included. Duration of the disease, family history, medications for UC, smoking, and extra-intestinal complications were noted for the UC group. Medical history, co-morbidities, and the other medications were also noted for both groups. Just before colonoscopic examination, the UC Mayo Scoring system (16) was used to assess disease activity. After colonoscopic examination, complete blood count, sedimentation rate, and C-reactive protein (CRP) were tested.

Table 1. Demographic and clinical characteristics of all participants

		Ulcerative Colitis n=95	Control n=38	p
Gender	Male	82 (86.3%)	35 (92.1%)	0.55**
	Female	13 (13.7%)	3 (7.9%)	
Age (year)		32.98±14.99	33.18±11.74	0.46*
Family History	Positive		8 (8.4%)	
	Negative		87 (91.6%)	
Smoking	Never smoked		59 (62.1%)	
	Quitted		13 (13.7%)	
	Still smoking		23 (24.2%)	
	No treatment		10 (10.5%)	
Treatment	ASA		63 (66.3%)	
	ASA±Steroids±Azothioprin		22 (23.2%)	
Disease Duration (month)			64.02±70.86	
Mayo Clinical Score			4.12±3.07	

*Mann Whitney U
**Chi-square
ASA: aminosalicylic acid

Assessment of endoscopic and histological activity

The endoscopic sub-score of the "Mayo Clinical Activity Index" was used for the endoscopic assessment of UC (Table 2). All colonic mucosal biopsy samples were fixed in 10% formaldehyde, paraffinized, and then 4 micrometer-thick slides were prepared. Samples were stained with hematoxylin and eosin, and examined under a Nikon Eclipse E600 microscope (China) by the same experienced pathologist as described previously (17). The Histological Activity Index values of the mucosal samples are shown in Table 2.

Immunohistochemistry assay

All sections, 4 micron thick of paraffin embedded tissue, were mounted on positively charged slides. Then, the slides were dried at room temperature overnight. The slides were de-paraffinized by a 12 h incubation in 56 °C. After standard rehydration procedures, antigen retrieval was secured and all other immu-

Table 2. Laboratory, endoscopic and histological findings of all participants

		Patient n=95 (Mean±SD)	Control n=38 (Mean±SD)	p
C reactive protein (mg/L)		9.1±13.93	3.09±2.32	<0.0001*
Sedimentation (mm/h)		18.99±16.54	9.84±8.46	<0.0001*
WBC (x10^3/mm^3)		8.5±3.17	7.11±2.16	0.012*
Platelets (x10^3/mm^3)		306.07±97.12	263.47±67.71	0.028*
Stomatin-like protein-2 score		5.77±2.16	4.74±2.18	0.003*
Prohibitin-2 score		1.2±2.4	3.61±4.68	<0.001*
Histological Activity	0	8 (8.4%)	37 (97.4%)	<0.0001**
	1	25 (26.3%)	0 (0%)	
	2	25 (26.3%)	1 (2.6%)	
	3	37 (38.9%)	0 (0%)	
Distribution	Absent	9 (9.47%)		
	Local Disease	74 (77.89%)		
	Pancolitis	12 (12.63%)		
Endoscopic Activity	0	14 (14.7%)		
	1 (Mild)	38 (40%)		
	2 (Moderate)	32 (33.7%)		
	3 (Severe)	11 (11.6%)		

*Mann Whitney U
**Chi-square
ASA: aminosalicylic acid

nohistochemistry staining steps were performed in a Ventana Benchmark XT Automated IHC/ISH slide staining instrument (Ventana Medical Systems; Tucson, AZ, USA), which used indirect biotin-avidin systems with human Slp-2 (sc-376165, Santa Cruz Biotechnology Inc.; Santa Cruz, CA, USA) and Pbh-2 (sc-133094, Santa Cruz Biotechnology Inc.; Santa Cruz, CA, USA) antibodies. Normal kidney tissue and endometrial adenocarcinoma tissue were used as positive controls. The concentration of primary antibodies (1:50) and incubation times (30 minutes) were the same for both antibodies. After rinsing, the slides were treated with biotinylated universal secondary antibody and avidin peroxidase (Ventana Medical Systems; Tucson, AZ, USA). Chromogen staining was done with diaminobenzidine (DAB). Finally, the slides were counterstained with Mayer hematoxylin and covered using a mounting medium and coverslip. The slides were evaluated under a Nikon Eclipse E600 microscope (China).

For Phb-2, a previously described method that was used for colon carcinoma by Chen et al. (12) was applied. Immunostaining intensity was classified as follows: lack of staining (0), mild staining (1), moderate staining (2), and strong staining (3) (Figure 1). The percentage of tissue staining positive was semi-quantitatively divided into five grades: <5% (0), 6–25% (1), 26–50% (2), 51–75% (3), and >75% (4). The immunohistochemistry score for each section was measured as the intensity score and positive staining percentage grade. Scores from 0 to 12 were obtained.

The percentage of Slp-2 positive cells was determined semi-quantitatively by assessing the entire tissue as described in a previous study for colon carcinoma (18). Expression of the protein was categorized as positive or negative and evaluated according to the percentage of cells stained: 0–5% (0), 6–49% (1), 50–74% (2), 75–89% (3), and 90–100% (4). The intensity of cell staining was assessed as 0 (no cell staining), 1 (weak), 2 (moderate), or 3 (strong). The two scores were multiplied to obtain a final score between 0–12.

Statistical analysis

Results are given as mean±standard deviation (SD). Data was analyzed using SPSS 15.0 (SPSS Inc.; Released 2006, SPSS for Windows, version 15.0, Chicago, IL, USA). Comparisons of means between groups were made using the Mann–Whitney U test, Chi-square, or the Kruskal Wallis test. The Spearman's rho correlation test was used when a linear relationship was present between two variables. The results were considered significant when $p<0.05$ was obtained.

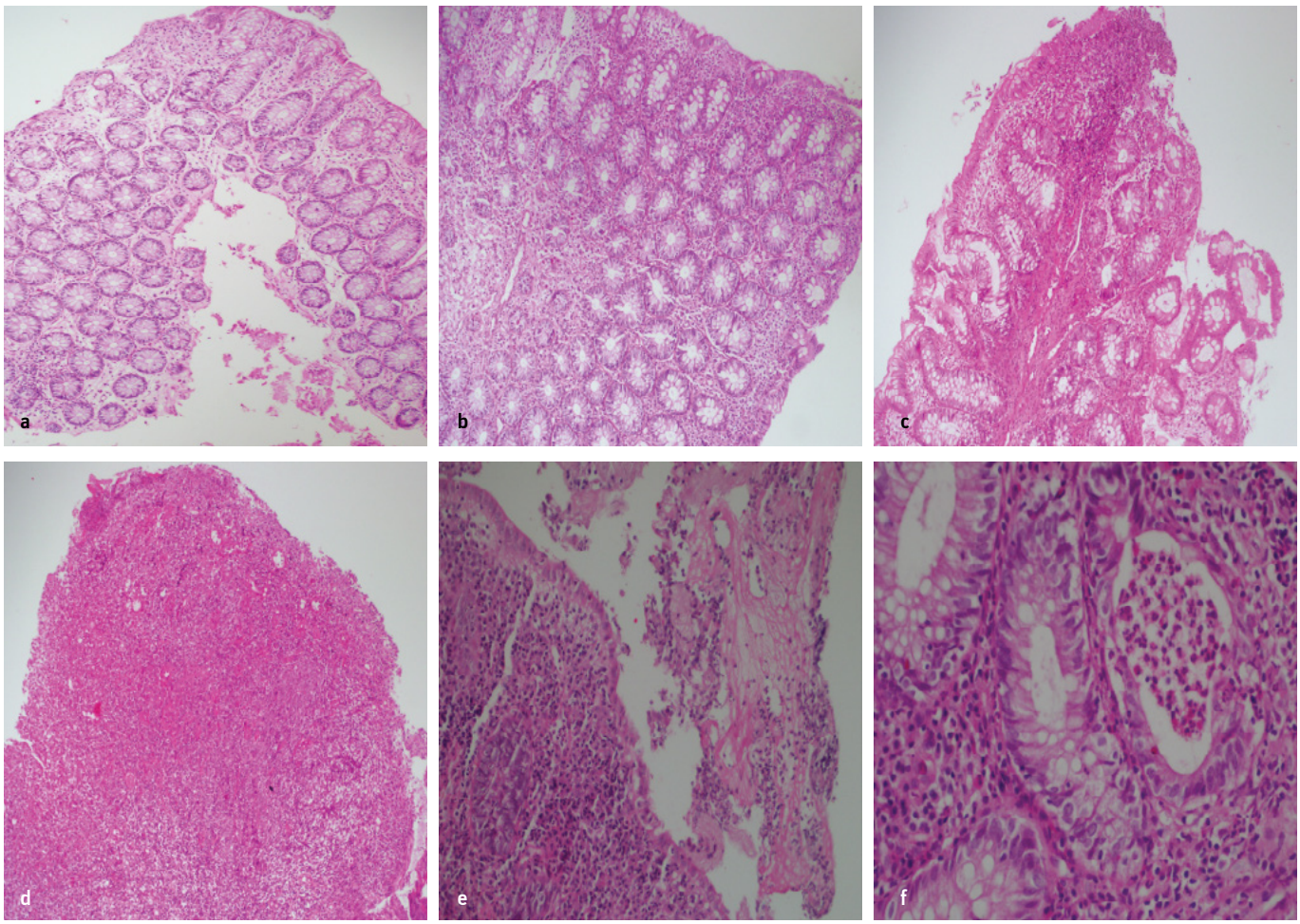

Figure 1. Immunohistochemical staining of colonic mucosal samples with prohibitin-2: mild (a), moderate (b), and strong (c), immunohistochemical staining of colonic mucosal samples with stomatin-like protein-2: mild (d), moderate (e), and strong (f)

RESULTS

No significant differences were found between patients with UC and the control group in terms of age and sex. Demographic and clinical characteristics of the UC and control groups are shown in Table 1. Laboratory, endoscopic, and histologic properties are shown in Table 2.

Phb-2 IHC scores were lower but Slp-2 scores were higher in the UC group compared to the control group, and the results were statistically significant (p<0.05, p=0.003, respectively, Table 2).

Table 3. Correlations between study parameters

			Phb-2 score	Slp-2 score	HAI	MCAS	EAI	Crp
Spearman's rho	Phb-2 score	r	1.000	.401**	.220*	.364**	.279**	.216*
		p	.	.000	.032	.000	.006	.036
	Slp-2 score	r	.401**	1.000	-.052	.067	.015	.012
		p	.000	.	.613	.516	.883	.907
	HAI	r	.220*	-.052	1.000	.690**	.714**	.382**
		p	.032	.613	.	.000	.000	.000
	MCAS	r	.364**	.067	.690**	1.000	.779**	.420**
		p	.000	.516	.000	.	.000	.000
	EAI	r	.279**	.015	.714**	.779**	1.000	.417**
		p	.006	.883	.000	,000	.	.000
	Crp	r	.216*	.012	.382**	.420**	.417**	1.000
		p	.036	.907	.000	.000	.000	.

HAI: histological activity index; MCAS: mayo clinical activity score; EAI: endoscopic activity index; Crp: C-reactive protein

Mayo clinical activity scores were positively correlated with histological activity and Phb-2 scores in all patients with UC (p<0.001, r=0.690 and p=0.001, r=0.364, respectively, Table 3 and Figure 2). Mayo clinical activity scores were also positively correlated with endoscopic severity and distribution (p=0.006, r=0.279 and p=0.002, r=0.317, respectively). However, there were no associations between family history, smoking, therapeutic agents, age, and Phb-2 scores.

A positive correlation was also found between Phb-2 score and CRP (p=0.036, r=0.216, respectively, Figure 2).

Slp-2 scores were higher in the patient group than in the healthy controls (Table 2). Moreover, Slp-2 and Phb-2 scores were positively correlated (p<0.001, r=0.401, Figure 2).

DISCUSSION

Assessment of UC activity is important for prognostication and therapeutic decisions (19). Oxidative damage plays a pivotal role in the pathogenesis of UC (20). Phb-2 and Slp-2 are structural proteins located in cellular membranes, especially in the mitochondrial inner membrane. Besides anti-inflammatory and anti-oxidative properties, Phb has also been shown to have anti-fibrotic activity in experimental cirrhosis and renal fibrosis (21-23). Slp-2 is a regulatory protein that provides the binding of Phb and other proteins to cardiolipin for optimal assembly of oxidative enzyme complexes in mitochondria (24). Slp-2 also modulates T-cell activation, which is an important process for

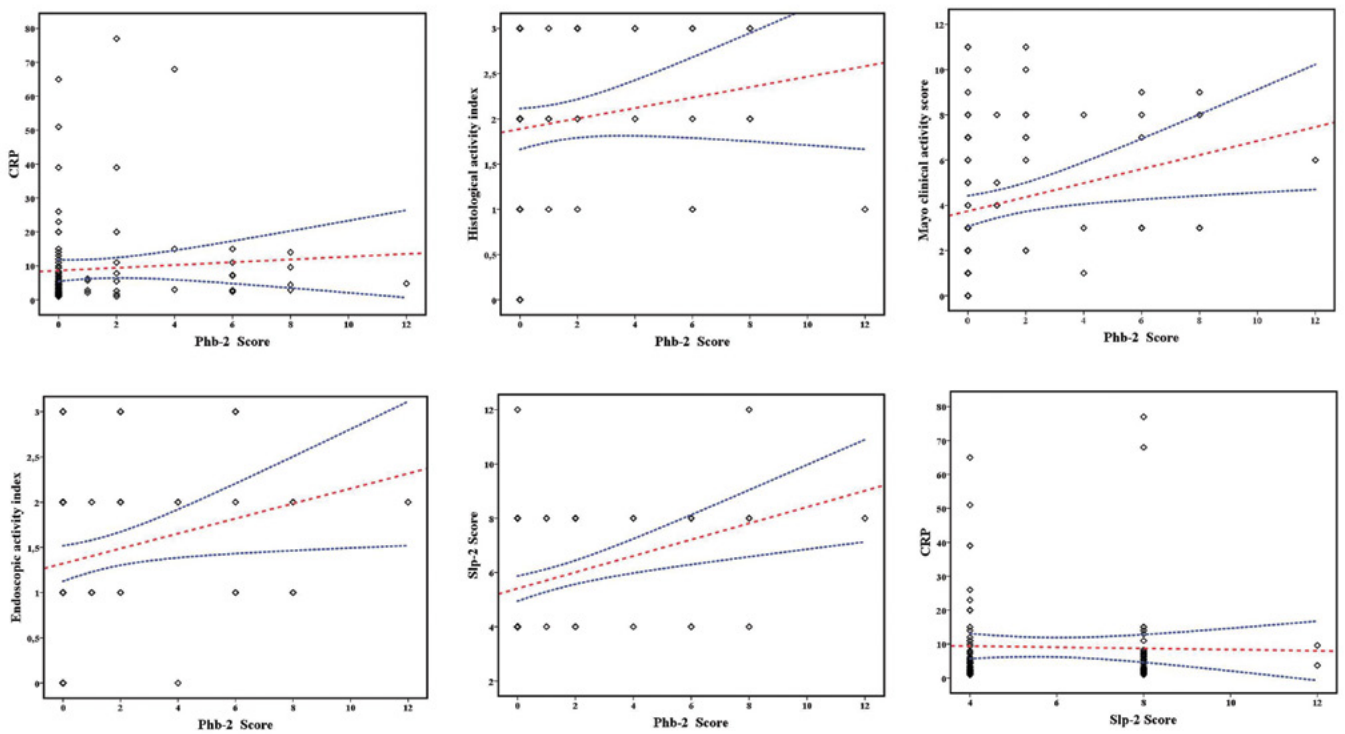

Figure 2. Correlation diagrams of prohibitin-2 and C-reactive protein, histological activity index, Mayo clinical activity score, endoscopic activity index, and stomatin-like protein-2 and correlation diagram of stomatin-like protein-2 and C-reactive protein

inflammation and UC (6). However, the activity of Slp-2 in UC has not yet been elucidated.

In our study, Phb-2 staining was prominent in epithelial cells of the colonic glands, especially in superficial epithelial cells, exposing a high antigenic potential at the side of the colonic lumen, but it was very weak in the stroma. This may reflect the immunological activity of epithelial cells and the protective specialty of Phb-2 (25-27). IHC staining of Slp-2 was diffuse both in epithelia and stroma, which indicated the structural property of this protein. In UC, oxidative enzymes decrease and mitochondrial dysfunction occurs (28,29). In a previous study, Hsieh et al. (8) examined the protein expression of colonic biopsy samples in patients with active and inactive UC, non-specific colitis, and normal mucosa. They found low protein expressions of Phb and some heat shock proteins in UC patients when compared to the other groups (8).

Arianne et al. (9) found low expression of Phb in the biopsy samples of patients with Crohn's disease compared to a healthy group. In this study, human epithelial cells were marked with cytokeratin 18 in vitro, which would be an indicator of cell lysis. Cytokeratin 18 levels were unchanged, which indicated that decreased Phb expression was not due to cell lysis. On the other hand, when human colonic epithelial cells were treated with hydrogen peroxide, which leads to oxidative stress, Phb and glutathione levels decreased. If the hydrogen peroxide was removed, the Phb and glutathione levels recovered (9).

Salmonella typhimurium was exposed to the colonic mucosa of both Phb transgenic rats (in which the Phb gene was transfected by plasmid into the rat DNA) and non-transgenic rats, so that *S. typhimurium* colitis occurred. In the transgenic group, histologic evaluations revealed less severe inflammation; in addition, weight loss was lower than in the other group after one week. As a result of the anti-inflammatory effect of Phb, microRNA levels of pro-inflammatory cytokines TNF-α, IL-1 β, and gamma interferon were also lower in the transgenic group (10).

Recent studies showed the anti-inflammatory and anti-oxidative properties of Phb (21-23,30). Is there a relationship between clinical, laboratory, endoscopic, and histological activities of UC and Phb? Our results showed that there is a positive correlation between endoscopic distribution and the severity of UC and Phb. Fluctuations of serum Phb levels may be a practical and non-invasive diagnostic method instead of colonoscopy, especially if the endoscopic procedure is contraindicated, or not desired, by the patient. The clinical activity assessment of UC is generally subjective. We found that clinical activity was positively correlated with Phb. For that reason, Phb levels may be an objective determinant of clinical activity.

Histological severity, especially if the score was 2 or 3, revealed more specific findings for the disease: a linear increase was observed in Phb levels. A similar correlation was also found between the common laboratory markers CRP and sedimentation rate.

Does chronic inflammation inhibit Phb, or does Phb deficiency lead to chronic inflammation? The answer is not clear. However, it is known that Phb directly inhibits Nf-kb activity (2). Intestinal epithelia may prevent inflammation by synthesizing Phb. Phb has been identified as a membrane-bound chaperon, or a kind of cellular stress protein, synthesized to prevent chronic inflammation and related oxidative stress (31). In the course of UC, different activity parameters are not generally consistent with each other. For example, colonoscopic severity may not correlate with clinical status. In this respect, Phb may be a valuable and reliable marker for correlating with all activity parameters.

Another aspect of Phb is its therapeutic potential. Previously, it has been shown that nanoparticle-based therapeutic delivery of Phb to the colonic epithelial cells ameliorates acute murine colitis (6,11). Correspondingly, the administration of Phb to the colonic mucosa of patients with UC may be a new therapeutic option.

Our results revealed that the scores of Slp-2 in UC patients were higher than those in the control group. This may be related to the high immunological activity of the disease. We also found a positive correlation between the two proteins, and this result may reflect the regulatory potential of Slp-2 for Phb-2. However, there was no correlation between the different activity parameters of UC and Slp-2. The probable cause of this result may be associated with immunomodulatory and immunosuppressive medications taken by most patients in the present study. Slp-2 has also been reported to be a therapeutic agent for autoimmune and inflammatory diseases (7). Further studies should be performed to evaluate the therapeutic potential of Slp-2 in UC.

The most important limitation of this study is the semi-quantitative method that we used for IHC evaluation. Integral optic densitometry along with spectroscopic computer programs might have been used to objectify the measurement. In addition, there were fewer female than male participants in both groups. It is unclear whether these proteins were affected by hormonal factors. The effect of hormonal factors on both Phb-2 and Slp-2 proteins should also be identified.

In conclusion, Phb-2 may be an emerging activity marker of UC, and further studies are needed to be performed to determine whether the modulation of these two proteins in colonic mucosa may be a therapeutic option in patients with UC.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Gülhane Military Medical Academy Haydarpaşa Training Hospital with the number of 2012-105 on 04.10.2012.

Informed Consent: Written informed consent was obtained from patients and control subjects who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - İ.K.; Design - İ.K.; Supervision - K.Ö., Y.Y., M.K.; Materials - İ.Y.; Data Collection and/or Processing - M.K., A.T., H.O.Ö.; Analysis and/or Interpretation - İ.Y.; Literature Review - A.T., İ.K.; Writer - İ.K., A.T., Y.B.; Critical Review - A.T., T.A.; Other - Y.B.

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